

8.2 Chemically Modified Nucleotides, Oligonucleotides and Nucleic Acids

Chemical modification of nucleic acids is not as straightforward as protein modification. The nonbasic "amines" of adenosine and guanosine are virtually unreactive with the amine-reactive reagents described in Chapter 1, and the other major reactive groups found in proteins — thiols, carboxylic acids and alcohols — are usually absent or not abundant in natural nucleic acids. Consequently, only a few techniques have been used for the direct labeling of DNA and RNA.^{1,2} Generally, nucleotides or oligonucleotides are labeled during chemical synthesis, then enzymatically converted into labeled nucleic acid polymers or directly used as primers or hybridization probes.³⁻⁹ For example, incorporation of amines or thiols during their synthesis permits nucleotides and oligonucleotides to be modified using the reagents in Chapters 1 and 2.

In addition to producing the widest assortment of nucleic acid stains (see Section 8.1), Molecular Probes supplies many of the most important dyes for nucleotide and oligonucleotide labeling, nucleic acid sequencing and direct or indirect nucleic acid modification. Our ChromaTideTM and FluoroTideTM products provide researchers with labeled nucleotides and oligonucleotides for enzymatic incorporation into nucleic acids, and our FluoReporter[®] Oligonucleotide Labeling and Purification Kits supply the reagents needed to reliably label and purify synthetic oligonucleotides without expensive equipment.

ChromaTide Labeled Nucleotides

Molecular Probes offers a series of uridine and deoxyuridine triphosphates, each conjugated to one of ten different fluorophores. The spectral diversity of the ChromaTide nucleotides (Table 8.2) gives researchers a great deal of flexibility in choosing a label that is compatible with a particular optical detection system or multi-color experiment. Our ChromaTide nucleotides⁹ contain a unique aminoalkynyl linker¹⁰ between the fluorophore and the nucleotide that is designed to reduce the interaction of the fluorophore with the nucleic acid and to make the hapten more accessible to secondary reagents (Figure 8.4). In addition to this four-atom bridge, several of these nucleotides contain a seven- to ten-atom spacer, which further separates the dye from the base.

Our newest ChromaTide nucleotides are the Oregon GreenTM 488, Rhodamine GreenTM and Texas Red-X conjugates of dUTP (C-7630, C-7629, C-7631) and the Rhodamine Green conjugate of UTP (C-7628). As compared to the corresponding fluorescein conjugates, the Oregon Green 488 and Rhodamine Green conjugates have similar fluorescence spectra but superior photostability (see Section 1.4). The Texas Red-12-dUTP (C-7631) has an emission spectrum in solution that is narrower and about 25% more intense than that of Texas Red-5-dUTP (C-7608).

Preliminary experiments have shown that the ChromaTide nucleotides are functional with a variety of nucleic acid modifying enzymes:

Table 8.2 Fluorophore labels for ChromaTide nucleotides.

ChromaTide Nucleotide Cat #	Fluorophore Label (Cat #, see Section n.n) *
C-7613, C-7614	BODIPY FL-X dye (D-6102, see Section 1.2)
C-7615, C-7616	BODIPY TMR-X dye (D-6117, see Section 1.2)
C-7617, C-7618	BODIPY TR-X dye (D-6116, see Section 1.2)
C-7611, C-7612	Cascade Blue dye (C-2284, see Section 1.7)
C-7603, C-7604	Fluorescein-X (F-2181, see Section 1.3)
C-7630	Oregon Green 488 dye (O-6147, see Section 1.4)
C-7628, C-7629	Rhodamine Green dye (R-6107, see Section 1.4)
C-7605, C-7606	Tetramethylrhodamine (T-1480 †, see Section 1.6)
C-7607, C-7608	Texas Red dye (T-353 ‡, see Section 1.6)
C-7631	Texas Red-X dye (T-6134, see Section 1.6)

* The catalog number for the labeling group and the Handbook Section number that contains its structure are indicated for each ChromaTide nucleotide. Fluorophores are attached to the terminal amine of the alkynyl spacer via a carboxamide linkage (arrow A in Figure 8.4) except for the following: † thiourea linkage, ‡ sulfonamide linkage.

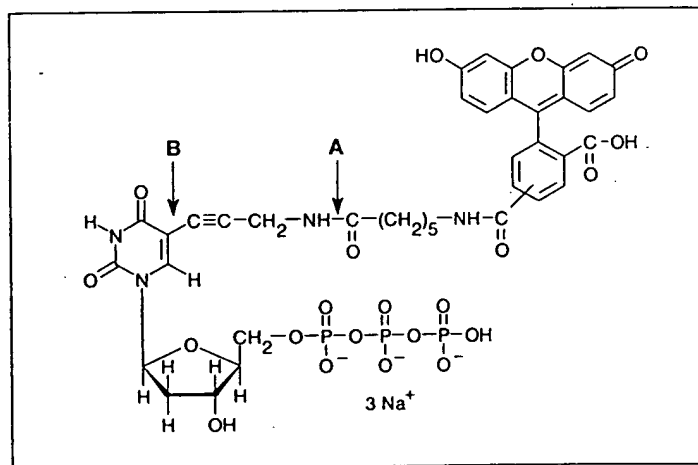


Figure 8.4 Structure of ChromaTide fluorescein-12-dUTP (C-7604). This structure is representative of our other ChromaTide labeled nucleotides. Fluorophore labels are attached via a four-atom aminoalkynyl spacer (between arrows A and B) to either deoxyuridinetriphosphate (dUTP) or uridinetriphosphate (UTP). Fluorophore labels for other ChromaTide nucleotides are indicated in Table 8.2.

- Taq polymerase in polymerase chain reaction (PCR) assays¹¹
- DNA polymerase I in primer extension assays
- Klenow polymerase in random primer labeling
- Terminal deoxynucleotidyl transferase for 3'-end labeling (see Color Plate 4 in "Visual Reality")
- SP6 RNA polymerase, T3 RNA polymerase and T7 RNA polymerase for *in vitro* transcription

ChromaTide nucleotides can also potentially be used as substrates in DNA sequencing reactions. Nucleic acids labeled with ChromaTide nucleotides can serve as probes for chromosome and mRNA FISH experiments, as well as for Southern, Northern, colony and plaque hybridization.

Labeled Oligonucleotides

Dyes for Nucleic Acid Sequencing

Molecular Probes manufactures most of the dyes that are used in nucleic acid sequencing and provides these in reactive forms for preparing conjugates. Because the electrophoretic separation step during sequencing is highly sensitive to the chemical structure of the fragments, the use of single-isomer labels is essential. In addition to providing high-purity reactive succinimidyl esters of the common FAM, JOE, TAMRA and ROX dyes,¹²⁻¹⁴ Molecular Probes prepares amine-reactive single isomers of carboxyrhodamine 6G (CR 6G) (Table 8.3). The 6-isomer of the CR 6G dye has been reported to have spectroscopic and electrophoretic properties that are superior to the JOE dye often used for automated DNA sequencing.¹⁵ Also, oligonucleotide conjugates of several of our BODIPY® dyes (see Section 1.2) have recently been reported to be very useful for DNA sequencing, in part because the dyes have a minimal effect on the mobility of the fragment during electrophoresis and also exhibit well-resolved spectra with narrow bandwidths¹⁶ (see Figure 1.2 in Chapter 1). The BODIPY dyes¹⁷ are all high-purity, pH-insensitive single isomers. Oligonucleotides labeled with multiple dyes that form excited-state energy transfer pairs have been shown to enhance the detection in sequencing applications that depend on the argon-ion laser for excitation.^{15,16,18,19}

Contact our Custom and Bulk Sales Department for information about custom synthesis of amine-reactive single isomers of our Rhodamine Green, Rhodol Green™ or other dyes^{20,21} or about availability of any of our reactive dyes in bulk.

FluoReporter Oligonucleotide Labeling Kits

Molecular Probes' FluoReporter Oligonucleotide Labeling Kits⁹ provide researchers with a convenient method for efficiently labeling oligonucleotides with a wide variety of our fluorophores and haptens. Labeling is not only economical, but easy and very reproducible. Our FluoReporter Labeled Oligonucleotide Purification Kit (F-6100) provides a simple way to purify the conjugates without resorting to HPLC or gel electrophoresis for purification.²²

We offer two types of FluoReporter Oligonucleotide Labeling Kits. The FluoReporter Oligonucleotide Amine Labeling Kits use stable succinimidyl esters to label amine-modified synthetic oligonucleotides, whereas the FluoReporter Oligonucleotide Phosphate Labeling Kits use proprietary coupling technology to conjugate aliphatic amines to 3'- or 5'-phosphate-terminated oligonucleotides in a single step. For unphosphorylated oligonucleotides, a 5'-phosphate can be added enzymatically with T4 polynucleotide kinase,

Table 8.3 Amine-reactive dyes for nucleic acid sequencing.

Cat #	Reactive Dye*	Handbook Location
C-2210	5-FAM, SE†	Section 1.3
C-6164	6-FAM, SE	Section 1.3
C-6127	5-CR 6G, SE	Section 1.5
C-6128	6-CR 6G, SE	Section 1.5
C-6171	6-JOE, SE†	Section 1.5
C-2211	5-TAMRA, SE	Section 1.6
C-6123	6-TAMRA, SE†	Section 1.6
C-6125	5-ROX, SE	Section 1.6
C-6126	6-ROX, SE†	Section 1.6
D-2184	BODIPY FL, SE‡	Section 1.2
D-6140	BODIPY FL, SSE	Section 1.2
D-6102	BODIPY FL-X, SE	Section 1.2
D-6180	BODIPY R6G, SE‡	Section 1.2
D-6117	BODIPY TMR-X, SE	Section 1.2
D-2222	BODIPY 564/570, SE‡	Section 1.2
D-2228	BODIPY 581/591, SE‡	Section 1.2
D-6116	BODIPY TR-X, SE	Section 1.2

* FAM = carboxyfluorescein; CR 6G = carboxyrhodamine 6G; JOE = carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein; TAMRA = carboxytetramethylrhodamine; ROX = carboxy-X-rhodamine; BODIPY = a substituted 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene derivative (see Figure 1.3 in Chapter 1); SE = succinimidyl ester; SSE = water-soluble sulfosuccinimidyl ester. † These are the most widely used isomers for DNA sequencing [Anal Biochem 223, 39 (1994); Nucleic Acids Res 20, 2471 (1992); Proc Natl Acad Sci USA 86, 9178 (1989)]. ‡ These BODIPY derivatives were recently reported to be useful for automated DNA sequencing, in part because the dyes have a minimal effect on the mobility of the fragment during electrophoresis and also exhibit well-resolved spectra with narrow bandwidths [Science 271, 1420 (1996)].

prior to use of the FluoReporter Oligonucleotide Phosphate Labeling Kits.

The amine-reactive haptens and fluorophores in most of our fifteen different FluoReporter Oligonucleotide Amine Labeling Kits contain aminohexanoyl spacers ("X") to reduce the label's interaction with the oligonucleotide and enhance its accessibility to secondary detection reagents. Our BODIPY FL-X, BODIPY TMR-X and BODIPY TR-X Kits (F-6082, F-6083, F-6084) contain reactive versions of our patented BODIPY fluorophores with emission properties similar to those of fluorescein, rhodamine 6G, tetramethylrhodamine and Texas Red dyes, respectively (see Figure 1.4 in Chapter 1). We also offer BODIPY FL, BODIPY R6G, BODIPY 564/570 and BODIPY 581/591 Kits (F-6079, F-6092, F-6093, F-6094), which contain reactive BODIPY dyes found to be useful for automated DNA sequencing.¹⁶ The BODIPY fluorophores exhibit high extinction coefficients, excellent quantum yields and a fluorescence emission that is quite photostable and insensitive to pH. The narrow absorption and emission bandwidths

of these BODIPY fluorophores make them particularly well suited to multicolor applications. The FluoReporter Oregon Green 488, Rhodamine Green-X, Rhodamine RedTM-X and Texas Red-X Oligonucleotide Amine Labeling Kits (F-6087, F-6088, F-6089, F-6091) contain some of our newest and most photostable dyes (see Sections 1.4 and 1.6). In addition to these kits, we offer FluoReporter Kits for labeling amines with fluorescein-X, tetramethylrhodamine, biotin-XX and DNP-X (F-6086, F-6090, F-6081, F-6085). The reactive dyes in some of the kits contain mixed isomers, and their oligonucleotide conjugates may resolve into two peaks in very high resolution separation techniques.

Conventional methods for modifying terminal phosphate groups require a multistep synthesis.²³⁻²⁵ In contrast, the FluoReporter Oligonucleotide Phosphate Labeling Kits permit the single-step covalent labeling of 3'- or 5'-phosphate-terminated oligonucleotides with cadaverine derivatives of the BODIPY FL, BODIPY TMR, or Texas Red fluorophores or biotin ligand (F-6096, F-6097, F-6099, F-6095) or with the ethylenediamine derivative of our Rhodamine Red dye (F-6098). The resulting phosphoramidate adducts have reasonable chemical stability, particularly in neutral solution. These kits can also be used to double-label radioactively labeled oligonucleotides or, in combination with T4 polynucleotide kinase, to fluorescently label oligonucleotides lacking a 5'-phosphate. In addition, we have found that the simple method provided in our FluoReporter Oligonucleotide Phosphate Labeling Kits can be used to label linear DNA restriction fragments, although such conjugates may require an alternative purification method.

The FluoReporter Oligonucleotide Amine Labeling Kits and FluoReporter Oligonucleotide Phosphate Labeling Kits provide sufficient reagents for five complete labeling reactions. Each FluoReporter Kit contains:

- Five vials of the amine-reactive or phosphate-reactive label, each sufficient for labeling 100 µg of amine-derivatized oligonucleotide
- Anhydrous dimethylsulfoxide (DMSO) for dissolving the reactive reagent
- Labeling buffer
- Detailed protocol for oligonucleotide labeling

Our FluoReporter Kits have been optimized for labeling oligonucleotides containing 18 to 25 bases but may be useful for labeling either shorter or longer oligonucleotides. Fluorescent, biotinylated or DNP-labeled oligonucleotides can be purified from the reaction mixture with our FluoReporter Labeled Oligonucleotide Purification Kit (see below) or with standard HPLC or gel electrophoresis methods. After purification, labeled oligonucleotides can serve as primers for DNA sequencing, DNA amplification or cDNA preparation and as probes for Northern and Southern blots, colony and plaque lifts and mRNA *in situ* hybridization experiments. Fluorescence anisotropy measurements can detect hybridization of fluorescent oligonucleotides in homogeneous solution.²⁶ RNA oligonucleotides are useful for probing RNA secondary structure, in combination with the dsRNA-specific RNase H and the ssRNA-specific RNase A and RNase T1.

FluoReporter Labeled Oligonucleotide Purification Kit

Purification of fluorescent, biotinylated or DNP-labeled oligonucleotides is made easy with our new FluoReporter Labeled Oligonucleotide Purification Kit (F-6100). The crude, labeled oligo-

nucleotide is simply precipitated with ethanol to remove the excess reactive reagent, adsorbed on the spin column, washed to remove any unconjugated oligonucleotide and then eluted with an elution buffer to yield the conjugate. Isolated yields for the combined conjugation and purification steps are usually >60%, and the products are typically >90% pure as determined by HPLC. This kit may be useful for purifying oligonucleotide conjugates of many of our other reactive dyes and haptens too. Oligonucleotide conjugates can be used for most procedures without additional purification. Each FluoReporter Oligonucleotide Purification Kit contains:

- Five spin columns
- Separate buffers for column equilibration, washing and elution
- Detailed protocol that has been tested with all of our FluoReporter Oligonucleotide Labeling Kits

Sufficient columns and buffers are provided for purification of five labeling reactions of 100 µg oligonucleotide each.

FluoTide Oligonucleotide Primers

Molecular Probes offers FluoTide oligonucleotide M13/pUC (-21) primers⁹ conjugated to fluorescein, Oregon Green 488, Texas Red-X, BODIPY FL, BODIPY R6G, BODIPY TMR and BODIPY TR dyes (F-3621, F-6677, F-6676, F-3622, F-7632, F-7633, F-7634). Our primer conjugates are prepared by attaching the dye to the 5'-(6-aminohexyl)-modified oligonucleotide, purified by preparative HPLC and packaged by optical density units measured at 260 nm. These primers are useful for automated single- or double-stranded sequencing of DNA fragments cloned into M13mp vectors and pUC or pUC-related plasmids.²⁷ They can also be used to synthesize hybridization sequences for probing blots, chromosome squashes, plaques, colonies and mRNA, as well as to generate a variety of probes for forensic and diagnostic applications.²⁸ The four BODIPY dye-labeled primers have well-resolved spectra with narrow bandwidths (see Figure 1.4 in Chapter 1), making them spectrally distinct from other fluorescently labeled primers and probes. Similar BODIPY dye-labeled primers have recently been reported to be very useful for DNA sequencing because the dyes do not produce the mobility artifacts exhibited by other dyes commonly used for DNA sequencing.¹⁶

Chemical Modification of Nucleic Acid Polymers

DNA and RNA are unreactive with most common chemical reagents, and special methods are necessary for their modification. Only a few general methods are available for modifying nucleic acid polymers.

Cytidine Residues

DNA and RNA can be modified by reacting their cytidine residues with sodium bisulfite to form sulfonate intermediates that can then be directly coupled to hydrazides or aliphatic amines.^{29,30} For example, biotin hydrazides (see Section 4.2) have been used in a bisulfite-mediated reaction to couple biotin to cytidine residues in oligonucleotides.³¹ Virtually any of the fluorescent, biotin or other hydrazides or aliphatic amines in Chapters 3 and 4, except possibly the BODIPY derivatives, can potentially be used in this reaction. The bisulfite-activated cytidylic acid can also be coupled to aliphatic diamines such as ethylenediamine.³² The amine-modified

DNA or RNA can then be modified with any of the amine-reactive dyes described in Chapter 1.

Phosphate Groups

Our FluoReporter Oligonucleotide Phosphate Labeling Kits provide the reagents and a protocol for the single-step modification of terminal phosphate residues of oligonucleotides or restriction fragments. Although phosphate groups of nucleotides and oligonucleotides are not very reactive in aqueous solution, their terminal phosphate groups can react with carbodiimides and similar reagents in combination with nucleophiles to yield labeled phosphodiester, phosphoramidate and phosphorothioate.³³ For example, it has been reported that DNA can be reacted quantitatively with carbonyl diimidazole and a diamine such as ethylenediamine to yield a phosphoramidate that has a free primary amine and that this amine can then be modified with amine-reactive reagents of the type described in Chapter 1.^{23-25,34} Fluorescent or biotinylated amines have been coupled to the 5'-phosphate of tRNA using dithiodipyridine and triphenylphosphine.³⁵ Wang and Giese have reported an apparently general method for labeling phosphates, including nucleotides, for capillary electrophoresis applications that employs an imidazole derivative prepared from our BODIPY FL hydrazide³⁶ (D-2371, see Section 3.2).

Abasic Sites

The biotinylated hydroxylamine ARP (A-6346) has been recently used to modify abasic sites in DNA — those apurinic sites and apyrimidinic lesions thought to be important intermediates in carcinogenesis.³⁷⁻³⁹ Once the aldehyde group in an abasic site is modified with ARP, the resulting biotinylated DNA can be detected with avidin conjugates (see Section 7.5).

Terminal Ribose Group of RNA

Selective oxidation of the 3'-end of RNA by periodate yields a dialdehyde. This dialdehyde can then be coupled with a fluorescent or biotin hydrazide reagent⁴⁰⁻⁴² (see Sections 3.2 and 4.2).

Specialized Methods

A few other specialized methods have been developed for nucleic acid modification. These include:

- Synthesis of DNA using fluorescent 2'- or 3'-acyl derivatives of uridine triphosphate and terminal deoxyribonucleotide transferase⁴³
- Use of a fluorescent iodoacetamide or maleimide, along with T4 polynucleotide kinase and ATP- γ -S (ATP with a sulfur in the terminal phosphate) to introduce a thiophosphate at the 5'-terminus of 5'-dephosphorylated RNA⁴¹ or DNA
- Introduction of 4-thiouridine at the 3'-terminus of DNA using calf thymus terminal deoxynucleotidyl transferase followed by treatment with ribonuclease and reaction with thiol-reactive probes^{44,45}
- Direct reaction of thiol-reactive reagents with 4-thiouridine residues in nucleic acids^{8,9,35,38,46,47}
- Direct reaction of amine- or thiol-reactive reagents with aminoacyl tRNA or thioacetylated aminoacyl tRNA^{35,48,49}
- Reaction of the X-base of tRNA with isothiocyanates⁵⁰ or replacement of other uncommon bases in tRNA by fluorophores⁵¹⁻⁵³

1. Kessler, C. in *Nonisotopic Probing, Blotting, and Sequencing*, L.J. Kricka, Ed., Academic Press (1995) pp. 41-109; 2. Kricka, L.K. in *Nonisotopic Probing, Blotting, and Sequencing*, L.J. Kricka, Ed., Academic Press (1995) pp. 3-40; 3. *Histochem J* 27, 94 (1995); 4. *Proc Natl Acad Sci USA* 89, 9509 (1992); 5. *Electrophoresis* 13, 542 (1992); 6. *Proc Natl Acad Sci USA* 86, 9178 (1989); 7. *Nucleic Acids Res* 16, 2203 (1988); 8. *Anal Biochem* 131, 419 (1983); 9. ChromaTide labeled nucleotides, FluoroTide labeled oligonucleotides and oligonucleotides prepared using our FluoReporter Oligonucleotide Labeling Kits are intended for research use only; use of labeled nucleotides and oligonucleotides for any other purposes or in any patented application may require licenses from Molecular Probes and other companies; 10. U.S. Patent No. 5,047,519 owned by E.I. DuPont de Nemours and Co.; 11. The PCR process is covered by patents owned by Hoffmann-LaRoche, Inc. Purchase of these products does not convey a license under these patents. Information about licenses for PCR can be obtained from Perkin-Elmer Corp. or Roche Molecular Systems, Inc.; 12. *Anal Biochem* 223, 39 (1994); 13. *Nucleic Acids Res* 20, 2471 (1992); 14. *Proc Natl Acad Sci USA* 86, 9178 (1989); 15. *Nature Med* 2, 246 (1996); 16. *Science* 271, 1420 (1996); 17. U.S. Patent Nos. 4,774,339; 5,187,288; 5,248,782; 5,274,113; 5,433,896; 5,451,663 and other U.S. and foreign patents pending; 18. *Proc Natl Acad Sci USA* 92, 4347 (1995); 19. *Anal Biochem* 231, 131 (1995); 20. *Anal Biochem* 207, 267 (1992); 21. Haugland, R.P. in *Optical Microscopy for Biology*, Herman, B. and Jacobson, K., Eds., Wiley-Liss (1990) pp. 143-157; 22. *FASEB J* 8, A1445 (1994); 23. *Anal Biochem* 218, 444 (1994); 24. *Biochem Biophys Res Comm* 200, 1239 (1994); 25. *Meth Mol Biol* 26, 145 (1994); 26. *Anal Chem* 67, 3945 (1995); 27. *Meth Enzymol* 101, 20 (1983); 28. *FEBS Lett* 351, 231 (1994); 29. *J Clin Microbiol* 23, 311 (1986); 30. *Biochemistry* 19, 1774 (1980); 31. *Biochem Biophys Res Comm* 142, 519 (1987); 32. *Biochem J* 108, 883 (1968); 33. *Nucleic Acids Res* 22, 920 (1994); 34. *J Chromatography* 608, 171 (1992); 35. *Biochemistry* 29, 10734 (1990); 36. *Anal Chem* 65, 3518 (1993); 37. *Biochemistry* 32, 8276 (1993); 38. *Biochemistry* 31, 3703 (1992); 39. *Biochemistry* 11, 3610 (1972); 40. *Bioconjugate Chem* 5, 436 (1994); 41. *Biochemistry* 30, 4821 (1991); 42. *Biochemistry* 19, 5947 (1980); 43. *Molekulyarnaya Biologiya* 11, 598 (1977); 44. *Anal Biochem* 170, 271 (1988); 45. *Nucleic Acids Res* 7, 1485 (1979); 46. *Biochemistry* 24, 692 (1985); 47. *J Mol Biol* 156, 113 (1982); 48. *J Am Chem Soc* 113, 2722 (1991); 49. *Eur J Biochem* 172, 663 (1988); 50. *Eur Biophys J* 16, 45 (1988); 51. *Eur J Biochem* 98, 465 (1979); 52. *Meth Enzymol* 29, 667 (1974); 53. *FEBS Lett* 18, 214 (1971).